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Mycobacterium bovis BCG-Mediated Protection against W-Beijing Strains of *Mycobacterium tuberculosis* Is Diminished Concomitant with the Emergence of Regulatory T Cells▽†

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Despite issues relating to variable efficacy in the past, the *Mycobacterium bovis* BCG vaccine remains the basis for new-generation recombinant vaccines currently in clinical trials. To date, vaccines have been tested mostly against laboratory strains and not against the newly emerging clinical strains. In this study, we evaluated the ability of BCG Pasteur to protect mice from aerosol infections with two highly virulent W-Beijing clinical strains, HN878 and SA161. In a conventional 30-day protection assay, BCG was highly protective against both strains, but by day 60 of the assay, this protection was diminished. Histological examination of the lungs of vaccinated animals showed reduced lung consolidation and smaller and more-organized granulomas in the vaccinated mice after 30 days, but in both cases, these tissues demonstrated worsening pathology over time. Effector T cell responses were increased in the vaccinated mice infected with HN878, but these diminished in number after day 30 of the infections concomitant with increased $CD4^+$ $Foxp3^+$ T cells in the lungs, draining lymph nodes, and the spleen. Given the concomitant decrease in effector immunity and continued expansion of regulatory $Foxp3^+$ cells observed here, it is reasonable to hypothesize that downregulation of effector immunity by these cells may be a serious impediment to the efficacy of BCG-based vaccines.

The global epidemic of disease caused by *Mycobacterium tuberculosis* continues unabated, with recent figures indicating approximately 8 million new cases of tuberculosis (TB) each year, with about 2 million deaths (5, 8, 9, 13, 31, 40). Among these infections, clinical isolates typed as belonging to the W-Beijing family of strains are becoming increasingly prevalent; in fact, it is believed that the Beijing genotype family is responsible for approximately 50% of TB cases in East Asia and now accounts for at least 13% of all isolates worldwide (4, 17, 29, 31, 33, 37, 38). The reason for the success of this family is unclear and is compounded by the suggestion that the current vaccine for tuberculosis, *Mycobacterium bovis* BCG, has actually selected for the emergence of this family (1).

Despite recent recommendations (10), we know little about the actual basic biology of the newly emerging clinical strains of *M. tuberculosis*. Only one W-Beijing strain, HN878, has been extensively studied with animal models to date (19–22, 36); the data obtained shows that this isolate is extremely virulent, at least in comparison with the modestly virulent laboratory strains H37Rv and Erdman. A major difference, as recently demonstrated (22), is that while all the above strains potently induce effector immunity 20 to 30 days after low-dose aerosol infection of mice, this is soon replaced in HN878-infected mice by the emergence of CD4 cells expressing Foxp3, with many of these staining in addition for the immunosuppressive cytokine interleukin-10 (IL-10).

Because vaccines for tuberculosis are usually tested against the laboratory strains, there is very limited information as to whether the existing BCG vaccine, or any of the new vaccine candidates in the current pipeline (2, 32), will be effective against the newly emerging clinical strains of *M. tuberculosis*, many of which appear to be highly virulent (23, 26–28). This issue is of particular importance given that the current lead vaccine candidates are recombinant versions of BCG (2). In the study reported here, mice were vaccinated with BCG and then infected with two clinical W-Beijing isolates of *M. tuberculosis*. In both cases, the outcome was the same. Thirty days after challenge, which is the conventional time at which protection is usually measured in this assay, both sets of vaccinated mice were significantly protected against the two W-Beijing strains. By day 60, however, this protection waned, and these animals exhibited increasingly severe lung pathology. Flow cytometric analysis of lung T cell populations showed a strong early effector T cell response, which then declined and was replaced by the steady influx into the lungs of $Foxp3^+$ regulatory T cells. In Kaplan-Meier analysis, the survival of the vaccinated mice was significantly prolonged in comparison to that of control animals, but these animals all eventually died. The data thus provide a new explanation for the spread of W-Beijing strains despite widespread BCG vaccination. Moreover, they raise serious questions about the strategy of using BCG, even recombinant versions, in new clinical trials in areas in which W-Beijing strains are highly prevalent.

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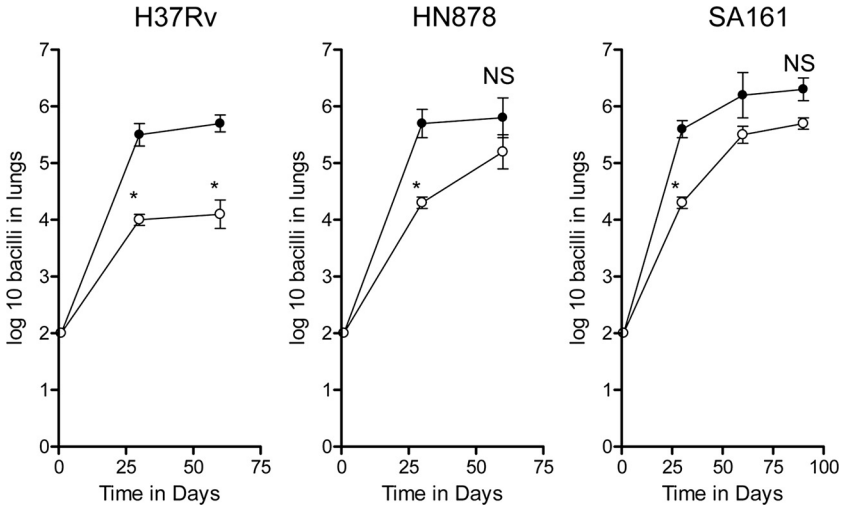

FIG. 1. Course of infections in the lungs of control and BCG-vaccinated mice. Data show the bacterial load in the lungs of control (closed circles) and BCG-vaccinated (open circles) mice infected by aerosol with *M. tuberculosis* strain H37Rv, HN878, or SA161. CFU were determined by plating serial dilutions of organ homogenates on nutrient 7H11 agar and counting CFU after 3 weeks of incubation at 37°C. Results are expressed as the mean ($n = 5$) of the bacterial load in each group expressed as $\log_{10}$ CFU (± standard error of the mean [SEM]). Data points indicated by asterisks were significantly different from results for saline controls. NS, not significant.

MATERIALS AND METHODS

Animals. Specific-pathogen-free female, 6- to 8-week-old C57BL/6 mice were purchased from the Jackson Laboratories, Bar Harbor, ME. They were maintained in the biosafety level 3 biohazard facility at Colorado State University (CSU) and were given sterile water and mouse chow. All experimental protocols were approved by the Animal Care and Use Committee of Colorado State University.

BCG vaccination. Animals were vaccinated subcutaneously with BCG Pasteur at a dose of 10^6 viable bacilli in 200 μl of sterile saline and then rested for 6 weeks prior to aerosol challenge. Control mice were injected with diluent only.

Experimental infections. Control and BCG-vaccinated mice were challenged by low-dose aerosol exposure to *M. tuberculosis* using a Glas-Col exposure device (Terre Haute, Inc.) calibrated to deliver 50 to 100 bacteria into the lungs of each mouse (12). The laboratory strain H37Rv was originally obtained from the Trudeau Institute collection (Saranac Lake, NY) and has been maintained at CSU by low-frequency passage. The clinical W-Beijing strain HN878 was kindly provided by B. Kreiswirth (PHRI, NJ), and the W-Beijing strain SA161 was kindly provided by K. Eisenach (University of Arkansas).

Course of infections. Bacterial burden in the lungs of infected animals ($n = 5$ animals) at the time points indicated in the figures was determined by plating serial dilutions of whole-organ homogenates on nutrient 7H11 agar and counting CFU after 3 weeks of incubation at 37°C in humidified air. The infection inoculum and day 1 lung bacterial counts were determined for all the bacterial strains tested by plating serial dilutions of inoculum or tissue homogenates on nutrient 7H11 agar and counting CFU after 3 weeks incubation at 37°C. No significant differences in the infection doses or the day 1 bacterial loads were present for any of the bacterial strains tested.

Flow cytometric analysis of cell surface markers. Single-cell suspensions were prepared as previously described (12). Briefly, the lungs were perfused with phosphate-buffered saline (PBS) containing heparin (50 U/ml; Sigma-Aldrich, St. Louis, MO) through the pulmonary artery, aseptically removed from the pulmonary cavity, and dissected in medium. The dissected lung tissue was further incubated with Dulbecco's modified Eagle's medium (DMEM) containing collagenase XI (0.7 mg/ml; Sigma-Aldrich) and type IV bovine pancreatic DNase (30 μg/ml; Sigma-Aldrich) for 30 min at 37°C. The digested lungs were then disrupted by gently pushing the tissue through a cell strainer (BD Biosciences, Lincoln Park, NJ). Red blood cells were lysed with ACK lysing buffer (NH_4Cl, $KHCO_3$, and EDTA), washed, and resuspended in complete DMEM containing 10% fetal bovine serum (Atlas Biologicals, CO). Total cell numbers were determined by flow cytometry using BD liquid counting beads, as described by the manufacturer (BD PharMingen, San Jose, CA). Cell suspensions from each individual mouse were incubated with monoclonal antibodies labeled with different fluorochromes at 4°C for 30 min in the dark. Monoclonal antibodies (MAbs) were used against the surface marker CD4 (clone Gk1.5, rat IgG2b,k), CD8 (clone 53-6.7, rat IgG2a,k), CD25 (PC61.5, rat IgG1,λ) CD44 (IM7, rat IgG2b,K), CD62L (MEL-14, rat IgG2a,K), CCR7 (4B12, rat IgG2a,k),CD69 (H1.2F3, Armenian hamster IgG), and CD95 (15A7, mouse IgG1,k) and isotype control rat IgG2a, rat IgG2b, rat IgG1, mouse IgG1, and hamster IgG were used in this study. These MAbs were purchased from BD Pharmingen or eBioscience (San Diego, CA) as direct conjugates to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), antigen-presenting cell (APC), eFluor450, Alexa Fluor 700, or Qdot800. All the samples were analyzed with a Becton Dickinson LSR-II instrument, and data were analyzed using FACSDiva v5.0.1 software. Cells were gated on lymphocytes based on characteristic forward- and side-scatter profiles. Individual cell populations were identified according to the presence of specific fluorescence-labeled antibodies. All the analyses were performed with acquisition of a minimum of 200,000 events.

For intracellular cytokine staining, lung, lymph node, or spleen cells were stimulated for 4 h with anti-CD3 and anti-CD28 in the presence of Golgi Stop (Becton Dickinson). After incubation, cells were harvested and first stained for cell surface markers as indicated above; thereafter, the same cell pellet were resuspended in permeabilization buffer using a commercial kit (Foxp3 staining buffer set; eBiosciences) and incubated for 30 min at room temperature. Cells were washed again and resuspended in Perm/Wash buffer containing labeled MAbs against Foxp3 (FJK-16S, Rat IgG2a), IL-17A (eBio17B7, Rat IgG2a,κ), and IL-17F (eBio18F10, Rat IgG2a), gamma interferon (IFN-γ) (XMG1.2, rat IgG1), and IL-10 (JES5-16E3, Rat TgG2b) and incubated for 30 min on ice. The cells were then washed twice and resuspended in PBS containing 0.05% sodium azide prior to analysis.

Histological analysis. Cranial lung lobes from each mouse were harvested and fixed with 4% paraformaldehyde in PBS. Sections were prepared and stained using hematoxylin and eosin.

Statistical analysis. Data are presented as representative of two independent experiments and are the mean values ($n = 5$) for replicated samples and duplicate or triplicate assays. The parametric Student *t* test, or Kaplan-Meier analysis, was used to assess statistical significance between groups of data.

RESULTS

Course of infection in control animals and in animals previously vaccinated with BCG. The course of the experimental infections is shown in Fig. 1. In each case, mice were vaccinated with BCG and exposed to aerosol infections 6 weeks later. Most vaccines to date have been tested against laboratory

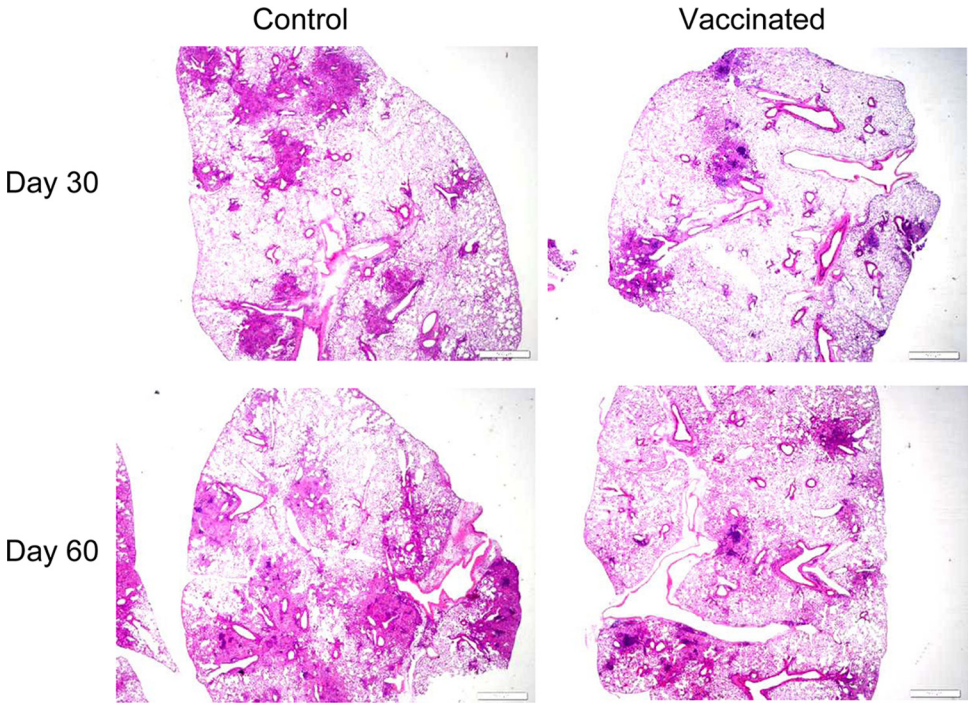

FIG. 2. Histopathology of the lungs of mice infected with W-Beijing strain HN878. Representative photomicrographs of lung sections taken at days 30 and 60 from control or vaccinated mice infected with strain HN878. Hematoxylin and eosin staining. Size bars, 500 μm.

strain H37Rv or Erdman with protection in $\log_{10}$ CFU conventionally measured about 30 days later. We show here, as anticipated, that mice vaccinated with BCG were more resistant than saline controls to H37Rv infection at day 30 ($P < 0.005$), with this resistance maintained at day 60 ($P < 0.01$). In a further experiment, we replaced the challenge infection with the W-Beijing strain HN878. At day 30, the protection observed for the vaccinated mice was 1.5 log ($P < 0.005$), but at day 60 of the experiment, the lung bacterial load was not significantly different from that of the controls. A similar result was obtained when we used the W-Beijing strain SA161 as the challenge, with protection highly significant at day 30 ($P < 0.0001$) but not significantly different from that of control animals on day 60 and day 90. Similar observations were seen after analysis of the bacterial load in the spleens (see Fig. S1 in the supplemental material).

Development of pathology in infected animals. Changes in lung pathology in mice infected with the two W-Beijing strains are shown in Fig. 2 and 3. As previously observed (22), the lungs of mice infected with HN878 exhibited progressive lesion development, and by day 60, much of the lung tissue in the control mice was grossly consolidated by inflammation gradually effacing the normal pulmonary architecture. Histologically, there were multifocal to coalescing aggregates of epithelioid macrophages with rare multinucleated giant cells, interspersed with aggregates of mature lymphocytes. In some animals, severe bronchopneumonia was seen, characterized by regionally extensive areas of coagulative necrosis that extended from major airways and effaced >75% of the tissue. By day 60, increasingly severe inflammation was seen, affecting greater than 40% of the lung tissues.

Overall, BCG vaccination slowed the progression of lesion development, and many inflammatory foci appeared more organized and dominated by large aggregates of lymphocytes. Vaccinated animals examined at 30 days postinoculation had foci of inflammation that were smaller than those in the control group and less extensive. Inflammation affected 10 to 15% of pulmonary parenchyma and was more lymphocytic than in the control animals. Vaccinated animals examined at day 60 had similar, lymphocyte-rich inflammation, but this had become more extensive, affecting up to 20% of the lung parenchyma.

A similar process was seen for mice infected with the W-Beijing strain SA161 (Fig. 3), in which the progression of disease from day 30 to day 60 was characterized by an increase in the size of the lesions and progression from multifocal to regionally extensive. Inflammation in these animals was characterized by variably sized foci of epithelioid macrophages with rare multinucleated giant cells and aggregates of lymphocytes. There was an obvious reduction in the numbers of lymphocytes as the infection progressed. From day 60 to 90, the patterns of inflammation were progressively more severe, with inflammatory foci coalescing and increased numbers of histiocytic cells filling alveoli. Progression of disease in BCG-vaccinated animals was initially far less severe, but by day 60, these foci were significantly larger, approaching the size of those seen for control animals. However, all vaccinated mice had more lymphocytes present within inflammatory foci relative to control animals, a difference that was most prominent at the later time points.

Kinetics of emergence of effector T cells. We harvested and purified cells from the lungs, draining lymph nodes, and spleens of vaccinated and control mice exposed to the two W-Beijing strains and analyzed these by flow cytometry. The

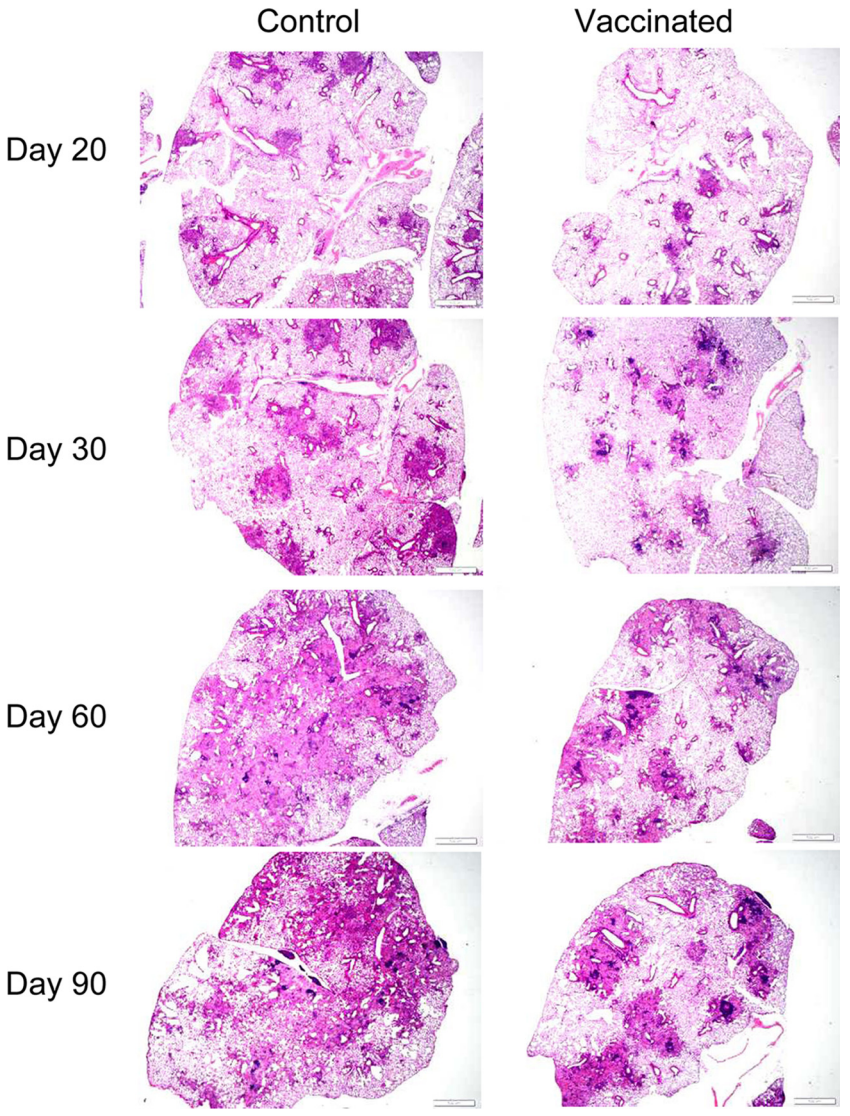

FIG. 3. Histopathology of the lungs of mice infected with W-Beijing strain SA161. Representative photomicrographs of lung sections taken at days 20 through 90 from control or vaccinated mice infected with strain SA161. Hematoxylin and eosin staining. Size bars, 500 μm.

kinetics of influx of effector T cells was determined, defined here as $CD4^+$ $CD44^{hi}$ $CD62L^{lo}$ IFN-γ^+ cells, and expressed as percentages of the total CD4 cells. In mice infected with HN878 and previously vaccinated with BCG, the influx of these cells in these tissues was 2-fold higher than in controls on day 30, but these percentages then declined by day 60 (Fig. 4). As with HN878, the percentage of effector cells in the lungs of both groups of mice infected with SA161 progressively declined during chronic infection after peaking at day 20 of the challenge infection.

Kinetics of emergence of $CD4^+$ $Foxp3^+$ and $CD4^+$ IL-17^+ T cells. Given our earlier demonstration that HN878 is a potent inducer of $Foxp3^+$ regulatory T cells (22), we then determined if the loss of protection observed above might be temporally associated with the arrival of this CD4 subset. In mice infected with HN878, we observed a progressive increase in the percentages of regulatory T cells, as anticipated (Fig. 5). In BCG-vaccinated mice, no evidence of any expansion or influx of these cells was seen by day 30 of the challenge infection, but by day 60, there was a significant surge in the influx of these cells. In the BCG-vaccinated mice infected with HN878 lymph nodes, percentages of these cells increased steadily, with no overt differences in percentages between the two groups. In the BCG-vaccinated mice infected with HN878 spleens, very low percentages of these cells were seen on day 30, but the percentages had increased in both groups by day 60.

Similar kinetic patterns were seen for mice infected with SA161. Again, there were much lower percentages of $CD4^+$ $Foxp3^+$ regulatory T cells in the vaccinated mice on day 20 and day 30 ($P < 0.02$), but these percentages then increased substantially by day 60. Similarly, the percentages of these cells were significantly lower ($P < 0.001$) in the mediastinal lymph nodes in the vaccinated mice at the early time points but then surged upwards, and a similar pattern was observed for the spleen.

Given the known role of CD4 cell secretion of IL-17 in

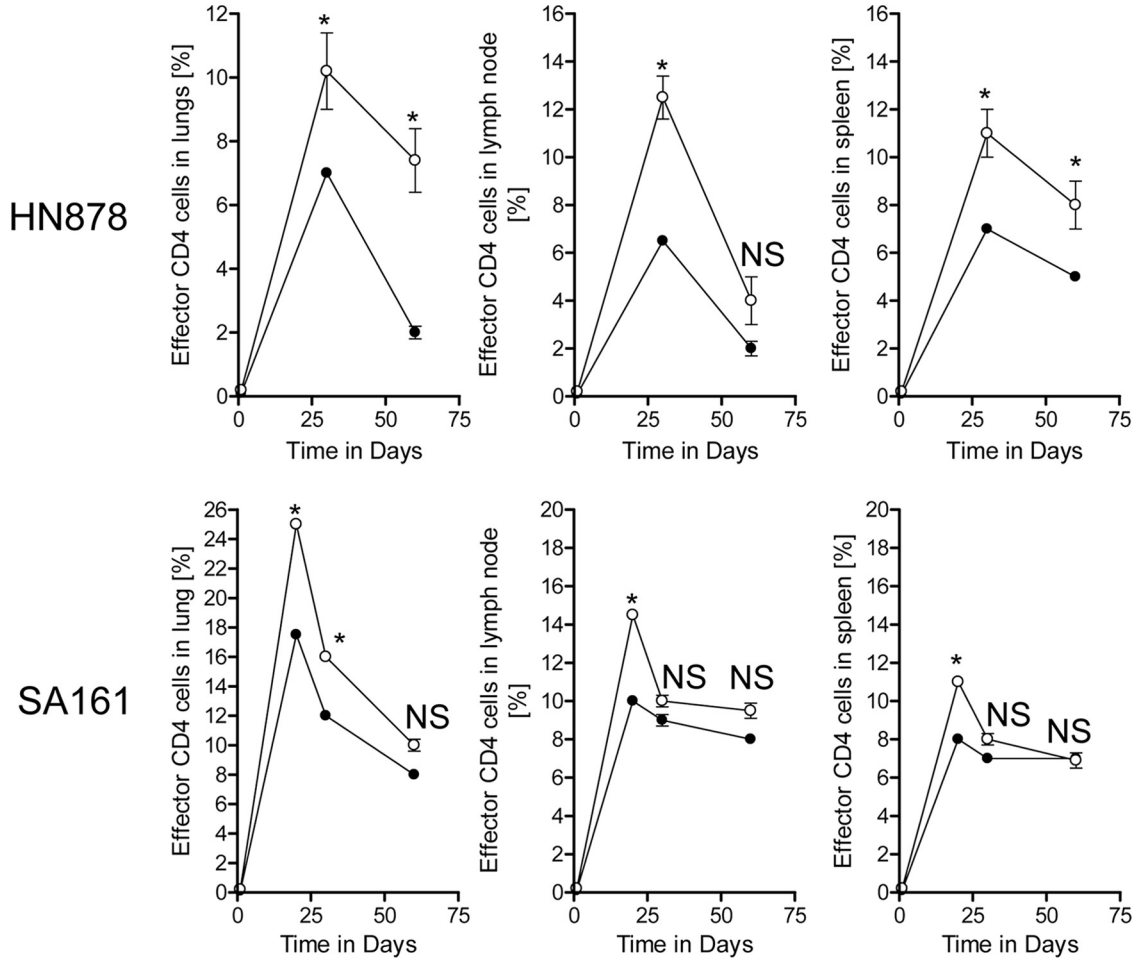

FIG. 4. Kinetics of influx of CD4 effector T cells in mice infected with strains HN878 and SA161. Lung cells obtained from control (closed circles) and BCG-vaccinated (open circles) mice were analyzed by flow cytometry. Effector cells were defined as those that stained positive as $CD4^+$ $CD44^{hi}$ $CD62L^{lo}$ IFN-γ^+ cells. The data are expressed as the mean percentage of total CD4 cells in each organ ± SEM (n = 5 mice per group). Data points indicated by asterisks were significantly different from results for saline controls. NS, not significant.

driving inflammatory responses (16), as well as the recent implication (15) that these cells play an important role in vaccinated mice, we also monitored the influx of CD4 cells in each group. In HN878-infected mice, the percentages of $CD4^+$ IL-17^+ cells increased progressively in both control and vaccinated animals in all three tissues analyzed (Fig. 6). Different kinetics were seen in mice infected with SA161, with a very large initial response in the lungs of the BCG-vaccinated animals. For reasons unknown, this profile was reversed in the draining lymph nodes and spleens, i.e., mice infected with SA161 showed increased percentages of $CD4^+$ IL-17^+ cells in the control lymph nodes and spleens.

BCG prolongs survival but does not ultimately protect against the W-Beijing strains. It was anticipated that the observed slowing of the inflammatory process seen with both sets of vaccinated mice would increase their survival, and this was subsequently observed in a Kaplan-Meier analysis of a separate set of mice. In two separate sets of studies, we consistently observed prolonged survival of vaccinated animals (Fig. 7). Among HN878-infected mice, control animals had a mean survival time of 85 days, consistent with our earlier studies (22), whereas vaccination increased mean survival to about 215 days. In mice infected with SA161, mean survival was 70 days, and this improved to about 170 days in mice given BCG.

DISCUSSION

The results of this study show that mice vaccinated with BCG and then challenged with *M. tuberculosis* H37Rv are significantly protected, as anticipated, and that this protection is sustained. In contrast, however, while mice infected with the W-Beijing strains HN878 and SA161 were equally well protected on day 30 post-aerosol infection, by day 60, the numbers of CFU in the lungs of control and vaccinated mice were not significantly different. Examination of the lungs of these animals showed an initial reduction in inflammation and lung consolidation and evidence of a better lymphocyte influx, as is usually seen when a vaccine is protective, but these elements were gradually lost as the disease process continued. Thus, while BCG vaccination clearly had an initially positive protective effect against the two W-Beijing strains tested here, this effect was transient and resulted only in a degree of prolonged

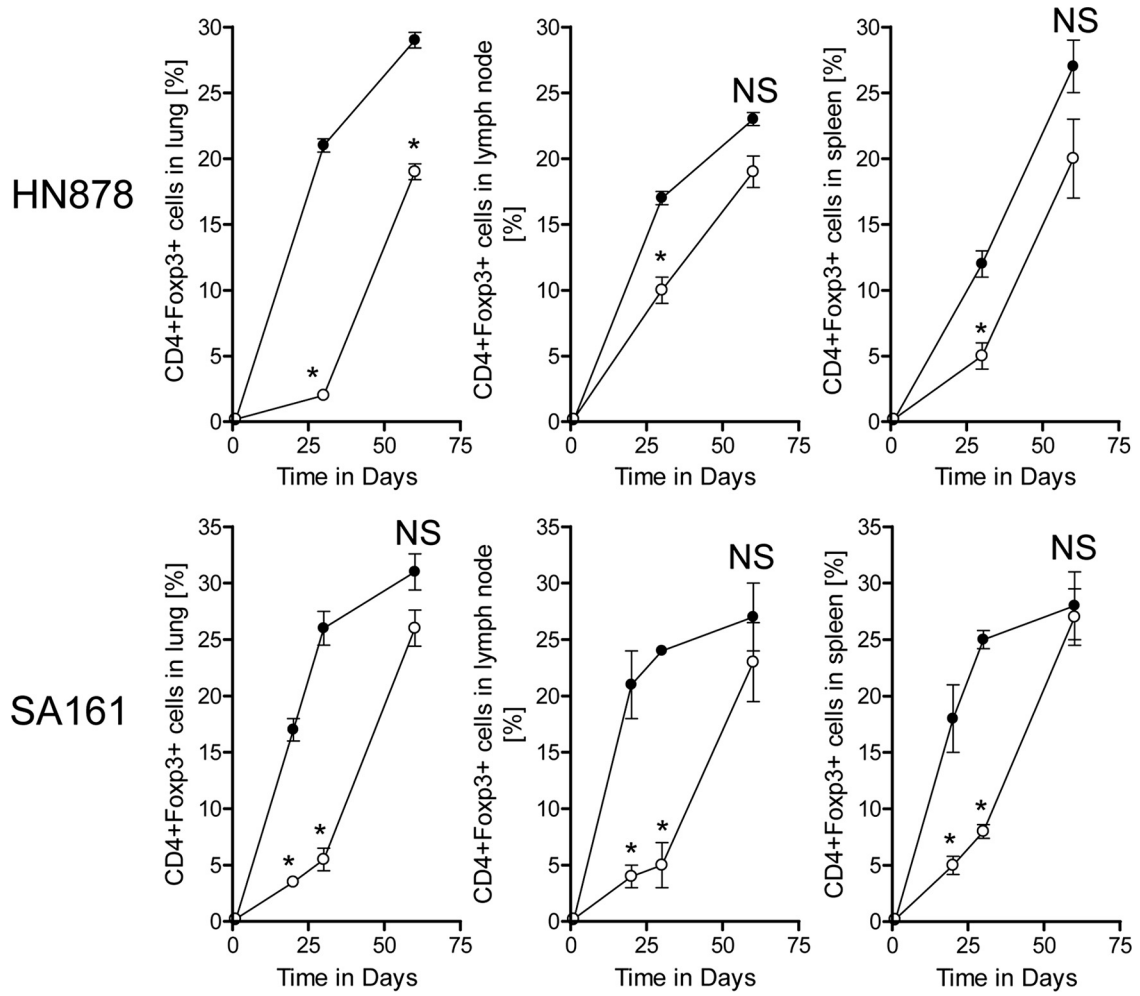

FIG. 5. Kinetics of influx of $CD4^+$ $Foxp3^+$ regulatory T cells in mice infected with strains HN878 and SA161. The total numbers of $CD4^+$ $Foxp3^+$ cells in the lungs of control (closed circles) and BCG-vaccinated (open circles) mice were analyzed by flow cytometry. The data are expressed as the mean percentage of total CD4 cells in each organ ± SEM (n = 5 mice per group). Data points indicated by asterisks were significantly different from results for saline controls. NS, not significant.

survival rather than long-lived protection. Many of the W-Beijing and other families of strains we have now tested in our laboratory are of equal or even far higher virulence than the HN878 and SA161 strains used here, and this raises the possibility that BCG-based vaccines may be ineffective in areas of the world where W-Beijing and potentially other families of high-virulence isolates are of increasing prevalence. Our serious concerns on this matter are discussed in more detail elsewhere (24, 25).

Analysis of effector T cell responses in animals with prior BCG vaccination and infected with HN878 and SA161 showed evidence of a considerable expansion of $CD4^+$ $CD44^{hi}$ $CD62L^{lo}$ IFN-γ^+ effector T cells, but this decayed significantly after 20 to 30 days. Given our earlier observation that mice infected with HN878 potently induce $CD4^+$ $Foxp3^+$ regulatory T cells (22), we further investigated this possibility and found evidence of a progressive increase in this cell population during the course of both W-Beijing infections, concomitant with worsening of the disease process. In mice infected with HN878 and SA161 that were first vaccinated with BCG, the expansion of $CD4^+$ $Foxp3^+$ cells was minimal on day 30, but thereafter began increasing and peaked on day 60. In SA161-infected mice, robust increases were observed for all the organs in control and BCG-vaccinated animals, with levels of such cells being only somewhat lower in the vaccinated mice on day 60. This may suggest that regulatory T cells are induced by the highly virulent, highly inflammatory SA161 infection very early on in the disease process, and indeed the capacity of the animal to rapidly generate such cells has recently been observed by others (30). Overall, these observations are thus consistent with the hypothesis that while BCG vaccination might reduce or delay the emergence or influx of $CD4^+$ $Foxp3^+$ regulatory T cells in response to these W-Beijing infections, it cannot prevent this from eventually happening.

As for TH17 cells, regarded by some studies as a counterbalance, results were more ambivalent. In HN878-infected mice, both control and vaccinated mice exhibited a steady influx of $CD4^+$ IL-17^+ cells, whereas this rate of cellular influx was initially much higher in the lungs of BCG-vaccinated mice infected with SA161. Studies by others (15) have suggested

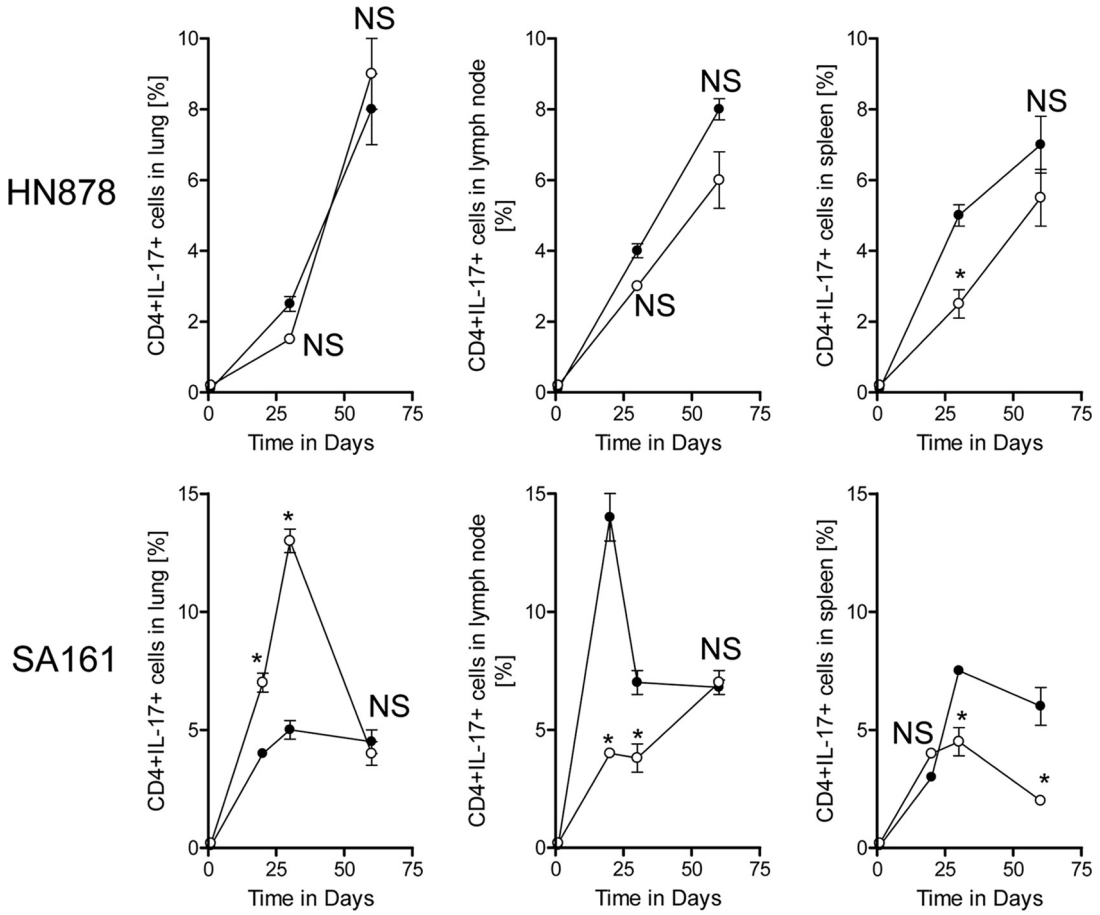

FIG. 6. Kinetics of influx of $CD4^+$ $IL\text{-}17^+$ T cells in control (closed circles) and BCG-vaccinated (open circles) mice were analyzed by flow cytometry. The data are expressed as the mean percentage of total CD4 cells in each organ ± SEM ($n = 5$ mice per group). Data points indicated by asterisks were significantly different from results for saline controls. NS, not significant.

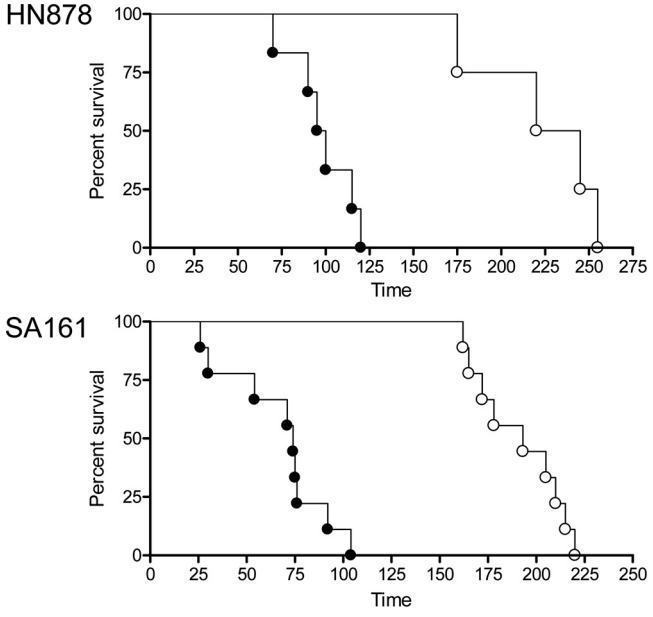

FIG. 7. Kaplan-Meier analysis of survival of mice after infection with HN878 or SA161. Data show the time to death of control (closed circles) and BCG-vaccinated (open circles) mice ($P < 0.0001$ in both cases, Kaplan-Meier analysis).

that vaccination induces a population of TH17 cells that facilitate the influx of memory immune effector T cells in tuberculosis by accelerating the needed chemokine response. If so, then this does not explain the low survival rates seen for BCG-vaccinated mice exposed to SA161 compared to those of animals infected with HN878, in which the TH17 response in the lungs was not potentiated by prior vaccination. Since both groups of animals were vaccinated with the same inoculum of BCG, then this result implies not only that individual isolates may differ in their capacity to trigger the lung TH17 response but also that a strong TH17 response, as seen to SA161, might induce increased $CD4^+$ $Foxp3^+$ expansion to counterbalance the proinflammatory effects of IL-17 cells and thus somewhat decrease the subsequent survival seen for these animals.

At this time, we can only conclude that the expansion or influx of $CD4^+$ $Foxp3^+$ regulatory T cells seems to parallel the decline of the more rapidly emerging effector T cell response to the two W-Beijing strains. Further studies involving regulatory T cell depletion are under way in our laboratory and will help further address this possibility. Our current working hypothesis is that the emergence of the $CD4^+$ $Foxp3^+$ population may be directly in response to lung inflammation and damage mediated by these two clearly highly virulent clinical strains, with suppression or at least interference with the ex-

isting protective effector T cell response as an unfortunate side effect. Given our observation (27, 28) that many of the newly emerging strains are capable of inducing severe lung damage, this is of great concern, especially if prior BCG vaccination as seen here can only slow this process down but not eventually prevent it.

Our observations here regarding the effects of BCG on the growth of clinically relevant isolates in the mouse lung are highly consistent with observations by others. Jeon et al. (14) tested a panel of clinical isolates in the mouse model and found that BCG was protective in mice infected with HN878 at 4 weeks, but this protection was then rapidly reduced. This study also examined the protective activity of BCG vaccination against several other clinical strains. In three examples, BCG protection remained relatively sustained after infection with the clinical strains, but protection declined substantially when other strains were used. Similarly, Grode et al. (11) demonstrated that BCG was only transiently protective against a modestly virulent W-Beijing strain and, interestingly, a recombinant BCG vaccine was more potent.

Our results are completely inconsistent, however, with the results of studies published by Sun et al. (34). In an earlier study (22), we clearly demonstrated that mice infected with HN878 by low-dose aerosol survived on average about 80 days, dying from the substantial lung pathology similar to that shown above. In the current study, we further demonstrated that BCG was able to slow the growth and subsequent lung pathology caused by the two W-Beijing strains, resulting in prolonged survival of these animals but no evidence of sustained protection. In contrast, Sun et al. (34) reported the mean survival of control mice infected with HN878 as being close to 300 days, an observation inconsistent with those in other reports demonstrating the high virulence ("hypervirulence") of HN878 (20, 22). In the Sun et al. study, BCG extended survival to ~375 days, and this was improved further to ~410 days in mice vaccinated with a lead recombinant BCG vaccine candidate.

The Sun et al. study and the induction of (negative regulator) CD8αα T cells in vaccinated macaques (18) support the usage of recombinant BCG vaccines, and these vaccines are the current focus of vaccine development today (2). As we have discussed elsewhere (25), one can make a series of arguments on why this may be a serious mistake. In addition to our data here that indicate that BCG-based vaccines will not establish immunity capable of overcoming the regulatory T cell response many of the virulent clinical strains seem to potently generate, we have also recently suggested other caveats. The first is that BCG seems to very poor at generating central memory (12, 24); the consequence of this is that while the effector memory response is faster than primary immunity, it still takes a finite time to be expressed (7) and this probably still allows virulent strains enough time to grow to the point that the regulatory T cell response can then get triggered. Thus, a vaccine candidate designed to induce central memory might be more effective, and one such example is a recombinant *M. smegmatis* ("Δike-plus"), which in a protective study (35) and in preliminary studies in our hands seems capable of inducing $CD44^{hi}$ $CD62L^{hi}$ IFN-γ^+ central memory T cells that expand and respond in as little as 7 days after *M. tuberculosis* challenge (I. M. Orme and W. R. Jacobs, unpublished observations); this potentially could be fast enough to contain virulent infections before $CD4^+$ $Foxp3^+$ cells become expanded.

A further issue regards the demonstration by Comas et al. that multiple families of *M. tuberculosis* have selectively conserved genes that encode T cell epitopes, including those expressed by immunodominant antigens (6). This is very concerning, because one explanation for this is that it ensures a potent T cell immune response, thus driving the granulomatous process but then also the caseous necrosis which eventually has the potential to lead to cavitation and hence transmission. If so, then overexpressing these antigens in a new recombinant BCG vaccine could potentially cause worsening clinical disease and increased transmission. Moreover, as we recently discussed (39), such recombinant vaccines would actually look superior in short-term animal model challenge experiments, especially if tested against the less-virulent laboratory strains. To avoid such problems, it might be wiser to focus on vaccines that express other protective antigens but not the primary dominant antigens, for example, ID93 (3).

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